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Symbiotic fungal associations in 'lower' land plants

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An analysis of the current state of knowledge of symbiotic fungal associations in 'lower' plants is provided. Three fungal phyla, the Zygomycota, Ascomycota and Basidiomycota, are involved in forming these associations, each producing a distinctive suite of structural features in well-defined groups of 'lower' plants. Among the 'lower' plants only mosses and *Equisetum* appear to lack one or other of these types of association. The salient features of the symbioses produced by each fungal group are described and the relationships between these associations and those formed by the same or related fungi in 'higher' plants are discussed. Particular consideration is given to the question of the extent to which root–fungus associations in 'lower' plants are analogous to 'mycorrhizas' of 'higher' plants and the need for analysis of the functional attributes of these symbioses is stressed.

Zygomycetous fungi colonize a wide range of extant lower land plants (hornworts, many hepatics, lycopods, Ophioglossales, Psilotales and Gleicheniaceae), where they often produce structures analogous to those seen in the vesicular-arbuscular (VA) mycorrhizas of higher plants, which are formed by members of the order Glomales. A preponderance of associations of this kind is in accordance with palaeobotanical and molecular evidence indicating that glomalean fungi produced the archetypal symbioses with the first plants to emerge on to land.

It is shown, probably for the first time, that glomalean fungi forming typical VA mycorrhiza with a higher plant (*Plantago lanceolata*) can colonize a thalloid liverwort (*Pellia epiphylla*), producing arbuscules and vesicles in the hepatic. The extent to which these associations, which are structurally analogous to mycorrhizas, have similar functions remains to be evaluated.

Ascomycetous associations are found in a relatively small number of families of leafy liverworts. The structural features of the fungal colonization of rhizoids and underground axes of these plants are similar to those seen in mycorrhizal associations of ericaceous plants like *Vaccinium*. Cross inoculation experiments have confirmed that a typical mycorrhizal endophyte of ericaceous plants, *Hymenoscyphus ericae*, will form associations in liverworts which are structurally identical to those seen in nature. Again, the functional significance of these associations remains to be examined.

Some members of the Jungermanniales and Metzgeriales form associations with basidiomycetous fungi. These produce intracellular coils of hyphae, which are similar to the pelotons seen in orchid mycorrhizas, which also involve basidiomycetes. The fungal associates of the autotrophic *Aneura* and of its heterotrophic relative *Cryptothallus mirabilis* have been isolated. In the latter case it has been shown that the fungal symbiont is an ectomycorrhizal associate of *Betula*, suggesting that the apparently obligate nature of the association between the hepatic and *Betula* in nature is based upon requirement for this particular heterotroph.

Keywords: bryophytes; cross inoculation; fungal symbioses; pteridophytes; ultrastructure

1. INTRODUCTION

It was evident to de Bary (1887) that intimate associations between organisms of dissimilar genotype are widespread in nature. He used the term 'symbiosis' to define such partnerships but perceived that the symbiotic condition could embrace a very broad range of relationships, some of which were of the antagonistic kind leading even to death of a partner, while in others both partners thrived in a mutually beneficial association. de Bary's view of the extent and nature of symbiosis has been supported by subsequent research. One of its main legacies is recognition of the need to establish, by experiment, the status of any apparently symbiotic association between organisms. However, progress towards understanding of function has generally lagged behind awareness of the extent of distribution of symbioses in biological systems. Nowhere is this more true than in the case of the symbiotic condition as demonstrated in 'lower' land plants. We have a broad base of knowledge of the distribution of these relationships in those groups of plants, which are the descendants of the original colonists of the terrestrial environment, but we know little of the status of these symbioses because experimental analyses have been scarce.

The present paper has two objectives. The first is to describe the present state of knowledge of the taxonomic

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Table 1. *The characteristics of the four most widespread mycorrhizal types found in 'higher' plants*

	kinds of mycorrhiza			
	VA	ectomycorrhiza	ericoid	orchid
fungi				
septate	−	+	+	+
aseptate	+	−	−	−
intracellular colonization	+	−	+	+
fungal sheath	−	+	−	−
Hartig net	−	+	−	−
vesicles	+ or −	−	−	−
arbuscules	+ or −	−	−	−
achlorophylly	− (? +)	−	−	+ [a]
fungal taxa	zygomycetes	basidio- and ascomycetes	ascomycetes	basidiomycetes
plant taxa	leptosporangiate ferns gymnosperms angiosperms	gymnosperms angiosperms	Ericales	Orchidaceae

[a] All orchids are achlorophyllous in the early seedling stages.
The structural characters given relate to the mature state, not the developing or senescent states.

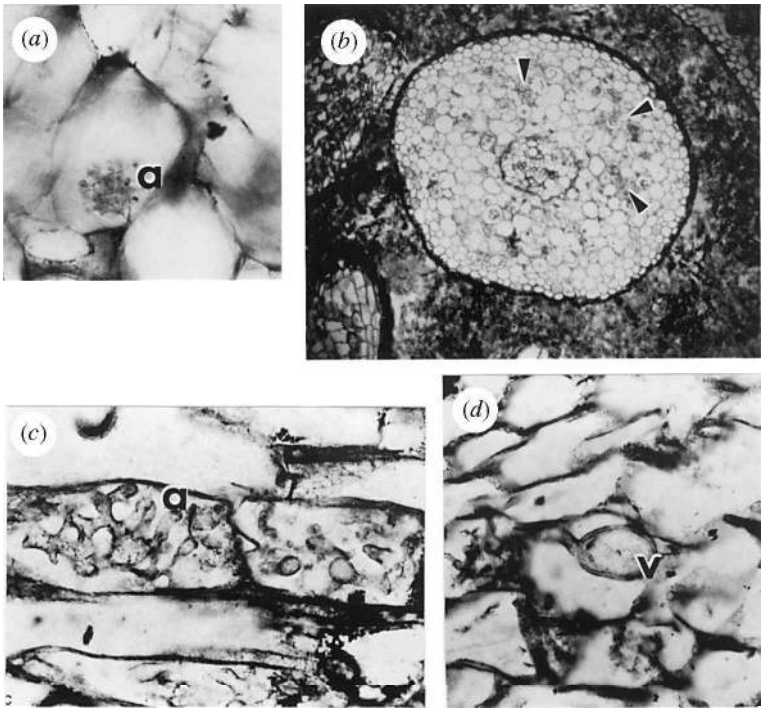

Figure 1. Fossil evidence for the occurrence of VA mycorrhizas in early land plants. (*a*) An arbuscule-like structure (a) in a cortical cell of *Aglaophyton* collected from Rhynie Chert rocks of Devonian age *ca.* 400 Myr BP. From Remy *et al.* (1994) with permission. Copyright National Academy of Sciences, USA. (*b*) Transverse section of a root of the cycad-related *Antarcticycas* obtained from rocks of Triassic age *ca.* 220 Myr BP in Antarctica. The central cortex contains areas in which the cells are occupied by mycorrhizal fungi (arrowed). (*c*,*d*) Higher magnification views of these cortical cells of *Antarcticycas* showing coarsely branched arbuscules (a) and a vesicle (v). (*b*–*d*) from Stubblefield *et al.* (1987*a*) with permission.

and structural features of those associations between symbiotic fungi and lower plants, which appear to be consistently present in nature. Second, it is intended to address the important question of the functional basis of the associations described.

The distinction between 'lower' and 'higher' plants is somewhat arbitrary, but for the present purpose is taken to lie between the supposedly 'primitive' land plants with large, sometimes achlorophyllous, gametophytes in the Bryopsida, Lycopsida, Equisetopsida or Pteropsida (only the Psilotales and Ophioglossales) and all those pteropsids, gymnosperms and angiosperms in which the gametophyte is more diminutive.

The roots of the majority of land plants are colonized by symbiotic fungi to form dual organs called 'mycorrhizas', the term being derived from the two Greek words 'mykes', fungus, and 'rhiza', root. Since, in the strictest sense, the underground axes and rhizoids of lower plants are not roots, any such fungal associations with them should not be called mycorrhizas. However, recent definitions of this symbiosis, for example that of Trappe (1996), see it in broader terms, referring to mycorrhizas as 'dual organs of absorption formed when symbiotic fungi inhabit healthy tissues of most terrestrial plants'. Under a definition of this kind, which does not specify roots, any healthy fungus-containing absorptive tissue of lower plants can legitimately be referred to as a mycorrhiza and more important questions concerning the extent to which the relationship is functionally as well as structurally analagous with these symbioses in higher plants can be addressed.

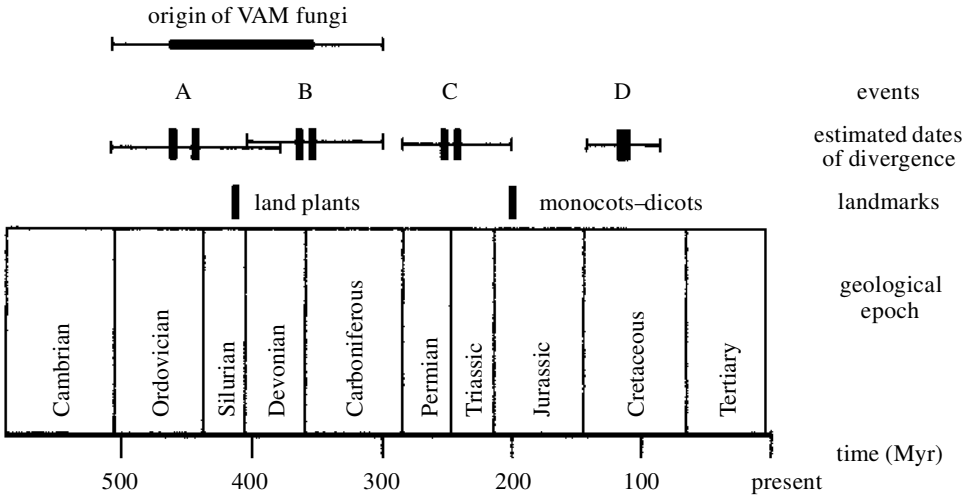

Figure 2. Demonstrating the synchronicity, revealed by molecular analysis, of the origins of VA mycorrhizal fungi (VAM) and land plants in the Bodovician–Silurian period. From Simon *et al.* (1993). Reprinted with permission. Copyright of Macmillan Magazines Ltd.

Table 2. *The characteristics of fungal associations found in 'lower' land plants*

	kinds of association		
	zygomycetous (cf. VA mycorrhiza)	ascomycetous (cf. ericoid mycorrhiza)	basidiomycetous (cf. orchid or ectomycorrhiza)
fungi			
septate	–	+	+
aseptate	+	–	–
intracellular colonization	+ or –[a]	+	+
vesicles	+ or –	–	–
arbuscules	+ or –	–	–
achlorophylly	– or +[b]	–	– or +[c]
bacteria-like organisms	+	–	–
fungal taxa	zygomycetes	ascomycetes	basidiomycetes
plant taxa	hornworts[d]	Jungermanniales[j]	Jungermanniales[k]
	Marchantiales[e]		Aneuraceae[l]
	Metzgeriales[k]		
	Lycopodium[f]		
	Botrychium[g]		
	Psilotum[h]		
	Gleicheniaceae[i]		

[a]In the protocorm of *Lycopodium cernuum*.
[b]Subterranean gametophytes of Psilotales, Ophioglossales, some Schizaeaceae.
[c]*Cryptothallus* only.
[d]Ligrone 1988.
[e]Ligrone & Lopes 1989; Ligrone & Duckett 1994.
[f]Duckett & Ligrone 1992; Schmid & Oberwinkler 1993, 1995.
[g]Schmid & Oberwinkler 1994.
[h]Peterson *et al.* 1981.
[i]Schmid & Oberwinkler 1995.
[j]Duckett *et al.* 1991; Duckett & Read 1995.
[k]This issue; Pocock & Duckett 1984.
[l]Ligrone *et al.* 1993.

The term mycorrhiza embraces four basic types of dual organ, each characterized by its own well-conserved structural attributes, which are a reflection largely of the particular fungi involved (table 1). Of the higher plants examined to date, over 90% have been shown to form associations of one or other of these types (Smith & Read 1997).

The type most widely distributed through the plant kingdom is that formed by zygomycetous fungi of the order Glomales, and referred to on the basis of two fungal structures, previously considered to be diagnostic for this type of symbiosis, namely 'vesicles' and 'arbuscules' as being vesicular-arbuscular (VA) or simply 'arbuscular' mycorrhiza (AM). It is, however, important to realize, particularly when considering fungal associations of lower plants, that arbuscules and vesicles are not necessarily produced in all plants colonized by glomalean fungi. It has been emphasized recently (Smith & Read 1997; Smith & Smith 1997) that there are two basic classes of VA mycorrhiza, which are named after the 'type species' of plant in which they were originally described by Gallaud (1905). These are the '*Arum*' and '*Paris*' types. The

'*Arum*' type forms in roots with extensive cortical intercellular spaces through which fungal hyphae grow before penetrating into the cortical cells to produce 'arbuscules'. The '*Paris*' type is found in species including, as described below, many 'lower plants', which lack well-developed systems of intercellular spaces. In this case, after penetration of the epidermis, growth of the fungus occurs almost exclusively in the intracellular position where distinct coils of hyphae are formed, sometimes without any arbuscules or vesicles.

Smith & Smith (1997) point out that because the distinctive fungal structures of the two types result primarily from the anatomy of the absorbing organ, the type of VA mycorrhiza that develops must be controlled genetically by the plant. Evidence in support of this view has been provided in a number of studies of higher plants. These show that a given species of glomalean fungus can produce an '*Arum*' type mycorrhiza in one host but a '*Paris*' type in another (Gerdemann 1965; Jacquelinet-Jeanmougin & Gianinazzi-Pearson 1983; Daniels-Hetrick *et al*. 1985).

Several lines of evidence suggest that these zygomycetous associations represent the archetypal mycorrhiza. Structures reminiscent of the vesicles seen in modern AM associations were seen and photographed by Kidston & Lang (1921) in underground axes of chert fossils from Devonian times. More convincing are the structures, also from Rhynie material and probably of *Aglaophyton* (figure 1*a*), which appear to be very similar to the intracellular 'arbuscules' formed by extant glomalean fungi (Remy *et al*. 1994). Two further features strongly support the view that these structures represent ancient 'mycorrhizas'. The first is that later fossils, for example from gymnosperm roots of the Carboniferous and cycad-like roots of the Triassic (figure 1*b–d*) (Stubblefield *et al*. 1987*a*,*b*,*c*), suggest a continuous presence of these structures through the course of land plant evolution. The second comes from analyses of substitutions in nucleic acid base sequences of the glomalean fungi forming this type of mycorrhiza in extant groups (Simon *et al*. 1993). These suggest an origin for these fungi between 460 and 350 Myr BP over a period which, according to the fossil record, land plants emerged (figure 2). One final feature pointing to the antiquity of zygomycetous associations is that these are by far the commonest type among lower land plants (table 2). In contrast, the ascomycetous ericoid and basidiomycetous orchid types are (i) far more restricted in their distribution, and (ii) confined to supposedly advanced taxa in both jungermannialean and metzgerialean hepatics.

A major factor selecting in favour of associations between glomalean fungi and early land plants may have been the geometrical inadequacy of the underground axes of autotrophs, which were making the transition from an aqueous to a soil-based system of nutrient supply (see Pirozynski & Malloch 1975). In extant groups of 'higher' plants, a relationship can be observed between responsiveness to these fungi and the fibrosity of their root systems. Thus, species with coarse root systems supporting few root hairs are usually more responsive to colonization than those with finely divided systems or having prolific root-hair development (Baylis 1972, 1975). It has been hypothesized that the dependence of early plants on colonization by organisms with more effective absorptive capabilities led to selective forces which favoured down-regulation of any mechanisms that would provide resistance to pathogenic attack (Vanderplank 1978). The observation that glomalean fungi penetrate and proliferate in the roots of so many species without apparently stimulating any of the physiological responses normally seen when plants are challenged by root pathogens (Bonfante & Perotto 1995; Gianinazzi-Pearson *et al*. 1996*a*,*b*; Kapulnik *et al*. 1996) lends support to this hypothesis. It suggests that compatibility was established before more advanced root systems were developed, and that lack of defence, even in species with fibrous root systems, is a genetically predetermined attribute in most families of land plants.

The advantages to the fungus of an ability to down-regulate or bypass the defences of a wide range of autotrophic species would be considerable. The opportunities for carbon acquisition, particularly in species-rich communities, are greatly increased, a feature that provides the resources required to generate a vigorous external mycelium. This, in turn, enables the fungus to locate and to colonize further root systems. Herein lies the challenge to those wishing to ascribe phylogenetic or functional significance to associations between glomalean fungi and their plant partners simply on the basis of occurrence of hyphae in the tissues. The presence of colonization can be simply a manifestation of the almost universal ability of these heterotrophs to penetrate the below-ground structures of autotrophic land plants. In each case, the nature of the relationship which ensues must be ascertained by experiment.

There are, of course, those families of higher plants in which root architecture is such that colonization by AM fungi appears to be essential for the acquisition of nutrients such as phosphorus, which are poorly mobile in soil (Merryweather & Fitter 1995; Smith & Read 1997). Here there is good reason to believe that coevolution of the partners has been essential for this survival and that the symbiosis is a mutualistic one. Proven instances of interdependence of these kinds are likely to be of phylogenetic as well as functional significance. In other families, however, particularly those consisting of plants with highly fibrous root systems, the colonization by the same fungi can have either neutral or antagonistic effects (Francis & Read 1995). The presence of the glomalean fungi here may reflect simply their ability to suppress host defence reactions and gain access to additional carbon supplies. It becomes obvious from these observations that records of the presence of colonization by AM fungi are not, by themselves, sufficient to enable either functional or phylogenetic significance to be inferred. This caution is as much relevant to consideration of fungal associations in lower plants as it is to the higher forms that have been the subject of more intensive study.

The fossil evidence indicates that ectomycorrhizas are a relatively recent form of the symbiosis, the first records being from Eocene rocks dated at *ca*. 80 Myr BP (Le Page *et al*. 1997). This type is characterized by the formation of a mantle or sheath of fungal mycelium over the outer surface of distal roots, by the lack of intracellular penetration of root cells and by the production of a very extensive network of vegetative mycelia in the soil around the roots.

The appearance of ectomycorrhizas in the fossil record is broadly coincident with the assumed date of origin of the homobasidiomycetes, which are the most commonly occurring symbionts of roots in families such as the Pinaceae, Fagaceae and Betulaceae, most members of which form this type of association. Because members of these families dominate the forest systems which cover a large proportion of the Northern Hemisphere, the ectomycorrhizal symbiosis plays a central role in global nutrient cycling processes.

While AM and ECM symbioses are the most widely distributed both through the plant kingdom and across the terrestrial surface, other forms of mycorrhiza become important in particular habitats. Thus the ericoid type, restricted to members of the order Ericales, is produced by ascomycetous fungi that invade the epidermal cells of the exceedingly fine 'hair-roots' in major genera such as *Calluna*, *Erica*, *Rhododendron* and *Vaccinium*. These plants characteristically dominate ecosystems at high latitudes or altitudes where low temperature inhibits decomposition and the cycling of key nutrients such as nitrogen and phosphorus. The fungi involved are known to play a major role in mobilizing such nutrients from the recalcitrant organic residues in which they are deposited (Read 1996).

The fourth distinctive mycorrhizal type is found exclusively in the largest of all terrestrial plant families, the Orchidaceae. All plants in this family are colonized by septate basidiomycetous fungi which penetrate cells of the roots where coarse coils of hyphae, called pelotons, are produced. Most, perhaps all, of the chlorophyllous orchids are colonized by a distinctive group of basidiomycetous fungi in the form genus *Rhizoctonia*. These can be isolated and sometimes induced to produce fruiting structures enabling their full taxonomic identity to be established. Among the genera identified to date, *Ceratobasidium*, *Thanatephorus* and *Sebacina* are important. In those orchids which retain the juvenile heterotrophic condition throughout the life cycle, such as the achlorophyllous species *Corallorhiza trifida* and *Cephalanthera austinae*, newly emerging evidence suggests that *Rhizoctonia* spp. have been replaced as symbionts by fungi, which at the same time are forming typical ectomycorrhiza with autotrophic trees (Zelmer & Currah 1995; Taylor & Bruns 1997; McKendrick *et al*. 2000).

The fungi involved in the four basic types of mycorrhiza seen in higher plants (table 1) are also known to form associations with distinctive groups of lower plants (table 2). In the remainder of this paper, their particular relationships with these groups will be described with a view to establishing the extents to which the associations found are structurally or functionally related to the mycorrhizas seen in 'higher' plants.

2. FUNGAL SYMBIOSES IN BRYOPSIDA

(a) *Taxonomic aspects*

Within the Bryopsida, interest lies chiefly in the hepatics and hornworts in which the occurrence of fungal associations possessing some of the structural attributes of mycorrhizas has been recorded frequently. It is a distinguishing feature of mosses, including *Sphagnum*, that associations of these kinds have not been recorded. Although there are scattered publications, dating from Peklo (1903), purporting to describe 'symbiotic' fungi in various moss tissues, both gametophytic and sporophytic (Mago *et al*. 1992), careful scrutiny of the data indicates that the fungi are confined to dead or moribund host cells and are thus almost certainly saprophytic or parasitic. This absence of fungi may in itself be a point of phylogenetic significance. It is certainly of physiological interest that mosses appear to resist colonization by mycorrhizal fungi so effectively. This lack of susceptibility places them alongside a select group of only a few higher plant families such as the Cruciferae, Caryophyllaceae and Polygonaceae, which are not normally colonized by these fungal symbionts.

The occurrence of symbioses between hepatics and fungi has been known for over a century. Schacht (1854) provided careful descriptions of fungi in the thalli of *Pellia* and *Preissia* and observed fungal colonization of rhizoids in *Marchantia* and *Lunularia*. Later, Janse (1897) provided illustrations of swollen rhizoid apices occupied by fungi in *Zoopsis*, a leafy jungermannialean hepatic. Bernard (1909) used the widespread occurrence of fungus–hepatic associations as the basis for his theory that vascular cryptogams were descended from mycotrophic bryophytes. A period of intensive light microscope (LM) analysis of these symbioses in *Pellia* (Ridler 1922; Magrou 1925) *Marchantia* (Burgeff 1938) *Lunularia* (Ridler 1923) *Aneura* (Gavaudan 1930) *Cryptothallus* (Malmborgh 1935) and *Calypogeia* (Nemec 1904; Garjeanne 1903) confirmed that these associations are a normal feature of hepatic biology. This early work is reviewed by Stahl (1949) and Boullard (1988). It enabled two broad types of association to be recognized, one involving aseptate fungi, often with arbuscules, and another formed by fungi with septate hyphae.

More recently, the use of combinations of histochemistry, LM and electron microscopy (EM) has provided further refinement of our understanding. These have confirmed that zygomycetous infections of the AM kind occur in *Phaeoceros* (Ligrone 1988) *Conocephalum* (Ligrone & Lopes 1989) and *Asterella* (Ligrone & Duckett 1994), as well as in members of the Metzgeriales, for example *Pellia* (Pocock & Duckett 1984). In addition, it has been possible to discriminate between the kinds of colonization formed by septate fungi. Thus a combination of ultrastructural (Duckett *et al*. 1991) and cytochemical (Duckett & Read 1991) approaches has revealed that the rhizoid infections occurring in leafy liverworts of the families Lepidoziaceae, Calypogeiaceae, Adelanthaceae, Cephaloziaceae and Cephaloziellaceae are caused by ascomycetous fungi possessing simple septa with Woronin bodies. In contrast, the septate hyphae occurring as endophytes in a few thalloid Metzgeriales such as *Aneura* and *Cryptothallus* (Ligrone *et al*. 1993) and in a few jungermannialean families (Boullard 1988; this paper) have been shown, by their possession of dolipore septa, to be basidiomycetes.

(b) *Structural and functional aspects of hepatic–fungus associations*

(i) *Zygomycetous associations*

A prerequisite for the analysis of the functional basis of any relationship between a micro-organism and its 'host' is that the putative partners be grown separately, then brought together to determine whether the naturally

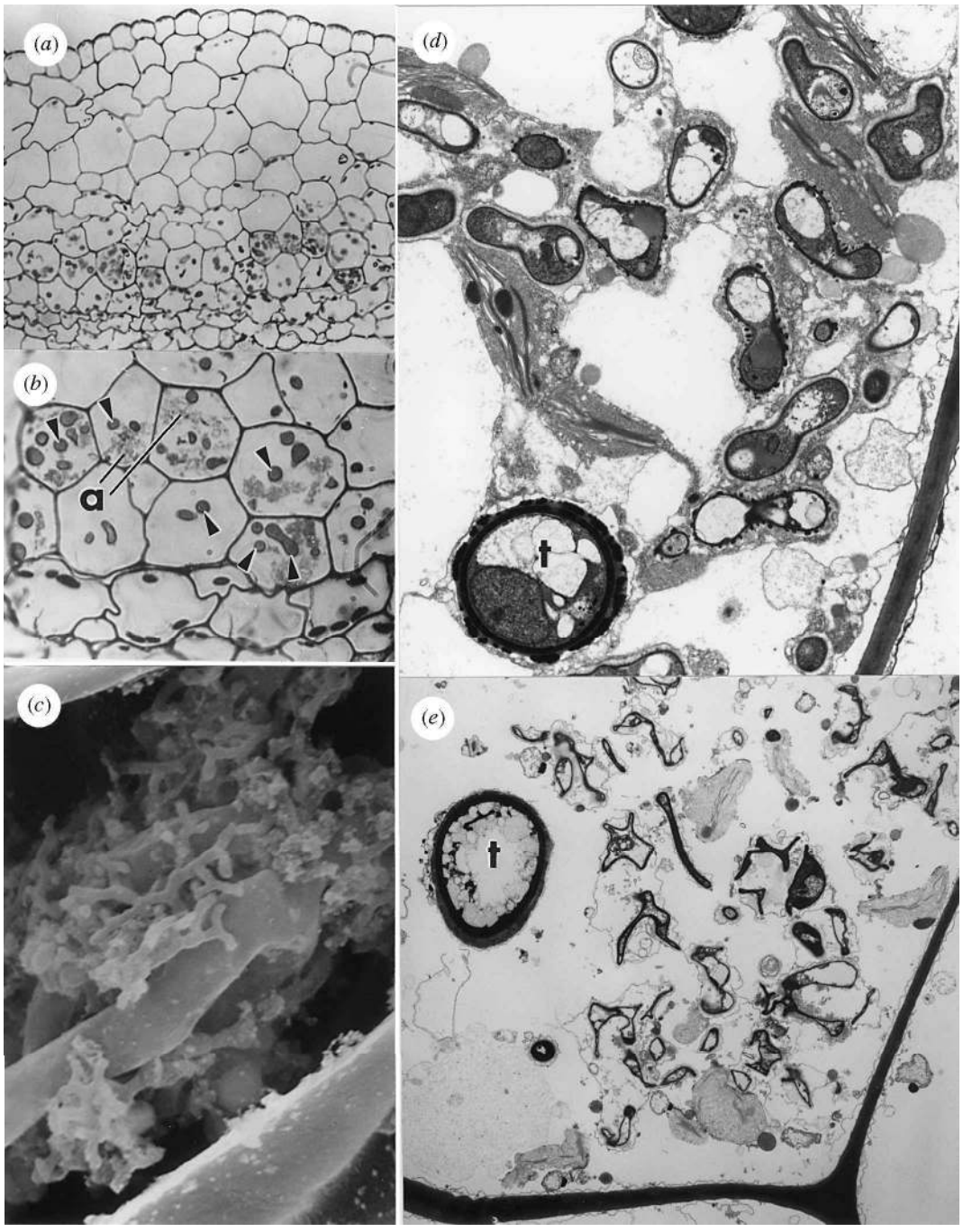

Figure 3. Thalli of *Pellia fabbroniana* colonized by a VA fungus spreading from *Plantago lanceolata*. (*a*,*b*) 1 μm toluidine-blue-stained sections showing the endophyte comprising trunk hyphae (arrows) and arbuscules (a) in the ventral thallus cells; (*a*) × 190, (*b*) × 500. (*c*) Scanning electron micrograph showing a trunk hypha and arbuscules; × 1900. (*d*) Transmission electron micrographs showing trunk hypha (t) healthy (*d*) and collapsed (*e*) arbuscules surrounded by host cytoplasm; (*d*) × 7100, (*e*) × 3400.

occurring symptoms and structures of the association are reproduced. This approach to the study of inter-organism interactions was established by Koch (1912) and has become central to diagnosis of pathogenesis and the aetiology of disease. Tests of what are now known as 'Kochs postulates' have only rarely been applied to the study of lower plant symbioses. They can be readily carried out in cases where the supposed microbial associate is culturable, as appears to be the case in many of the ascomycetous and basidiomycetous associates of 'lower plants', but are more difficult to achieve in the case of zygomycetous infections because the glomalean fungi, which are putatively the causal organism, cannot be grown free of their hosts. Spores of these fungi can readily be obtained from soil or 'pot cultures', but they are not the propagules of choice in studies of infectivity because of the low vigour or 'inoculum potential' sensu Garrett (1970) of the vegetative hyphae which they produce.

Study of functional aspects of the relationship between *Pellia* and its fungal endophytes was pioneered by Magrou (1925). He sowed spores on to soils bearing propagules of AM fungi and produced young gametophytes that were colonized at an early stage of development. Magrou (1925) reported localized cell death and inhibition of

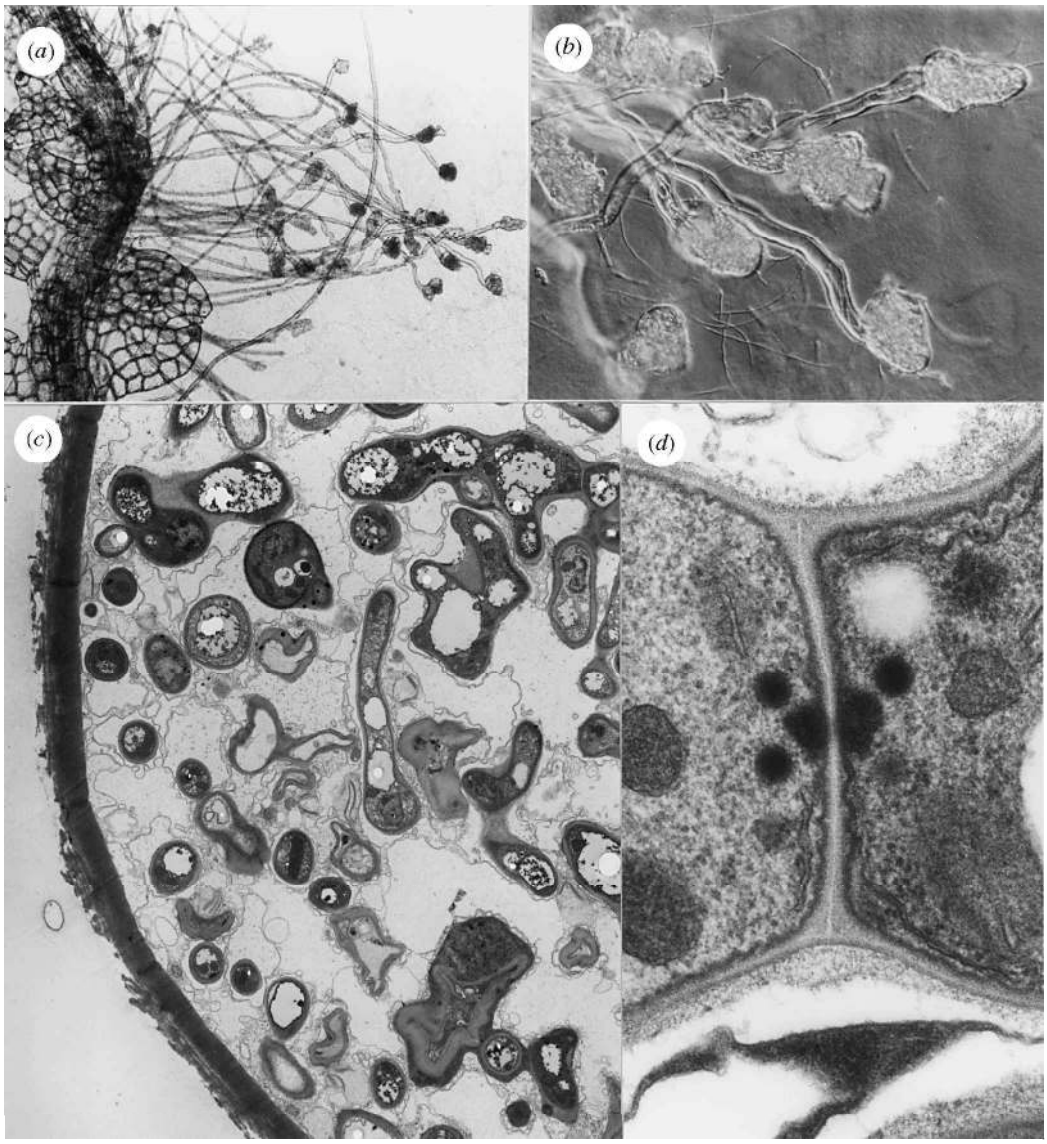

Figure 4. Swollen-tipped rhizoids of *Cephalozia connivens* after experimental inoculation with the ericoid mycorrhizal fungi *Hymenoscyphus ericae*. (*a*,*b*) Light micrographs; (*a*) × 55, (*b*) × 230. (*c*) Transmission electron micrograph showing numerous hyphae surrounded by host cytoplasm; × 4300. (*d*) Detail of the fungus showing a simple septum with Woronin bodies; × 58 500.

thallus development in colonized plants. In naturally occurring plants he noticed the non-uniform nature of infections, which were always absent from the thallus in the vicinity of sex organs or developing sporophytes. Only after dehiscence of the capsule did extensive invasion of mature tissues take place, this being associated with the production of vesicles and arbuscules.

An approach which mimics more effectively the infection process in nature, was developed by Francis & Read (1995) to investigate the impact of vigorous VA mycelial networks on the development of higher plants, and to provide distinction between true 'hosts' to these fungi and 'non-hosts'. In this, plants of species known to be 'hosts' are grown in a natural substrate with or without their glomalean associates. In the case of the pre-infected mycorrhizal plants, the fungus is allowed, by growing through a mesh of fine pore size, to colonize adjacent root-free-soil. The response of seedlings of test plants introduced to this soil and thus exposed to the fungus or with 'natural' inoculum potential, is compared with that obtained in the same substrate lacking the VA mycelium. This approach has been used to ask two basic questions concerning zygomycetous infections of thalloid hepatics. First, do the coarse VA endophytes which are the normal colonists of higher plant roots satisfy the requirements of Koch's postulates by colonizing hepatics and reproducing typical symptoms of infection? Second, does any infection observed stimulate growth or phosphorus content of the hepatic in a manner normally seen in higher plants when a compatible association forms? Our experiments thus far indicate that VA fungi, colonizing seedlings of *Plantago*, will spread into axenically grown thalli of *Pellia* (figure 3) where they produce trunk hyphae, arbuscles and vesicles apparently similar to those seen in wild specimens. It remains to be demonstrated whether these infections are functionally similar to those in higher plants.

(ii) *Ascomycetous associations in hepatics*

A range of leafy hepatics in the Cephaloziaceae and related Jungermanniaceae develop swollen rhizoids

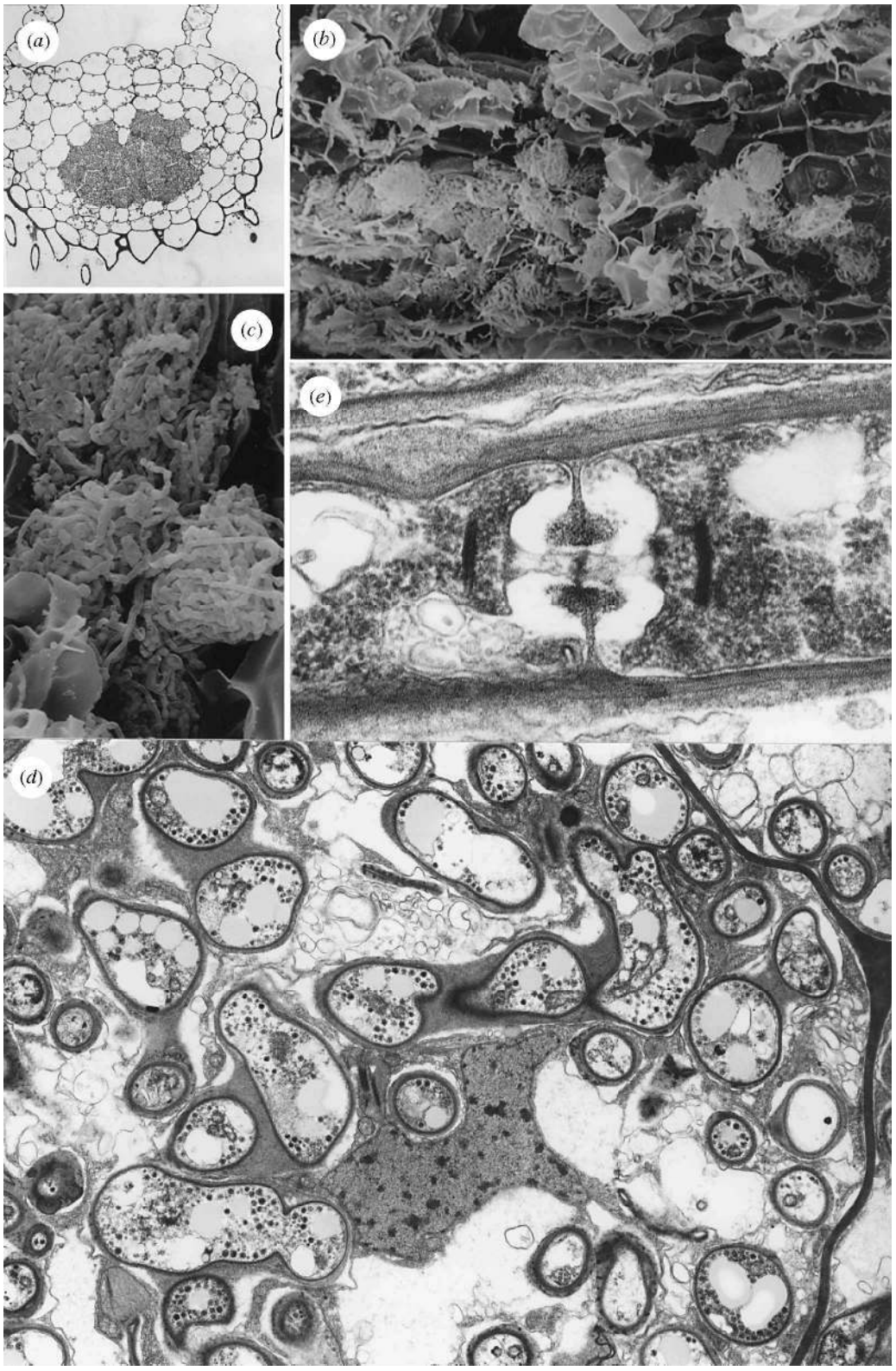

Figure 5. The basidiomycetous endophyte in the leafy hepatic *Southbya tophacea*. (*a*) 1 μm toluidine-blue-stained transverse section showing fungus-containing cells in the centre of the stem; × 130. (*b*,*c*) Scanning electron micrographs showing hyphal coils in the inner stem cells; (*b*) × 280, (*c*) × 5500. (*d*) A mass of fungal hyphae within a host cell with healthy cytoplasm; × 9500. (*e*) Dolipore with imperforate parenthosomes; × 59 000.

packed with fungal hyphae (Duckett *et al*. 1991; Pocock & Duckett 1985; Williams *et al*. 1994). An EM study of rhizoids in the Cephaloziaceae revealed the presence of simple septa and Woronin bodies in the hyphae, characteristic of ascomycetous fungi (Duckett *et al*. 1991). In addition, the hyphae showed high affinity for the fluorescent dye 3,3′-dihexyloxacarbocyanine iodide, a further distinguishing feature of the ascomycetes (Duckett & Read 1991). Unlike VA fungi, these can be readily isolated and grown on water agar (Duckett &

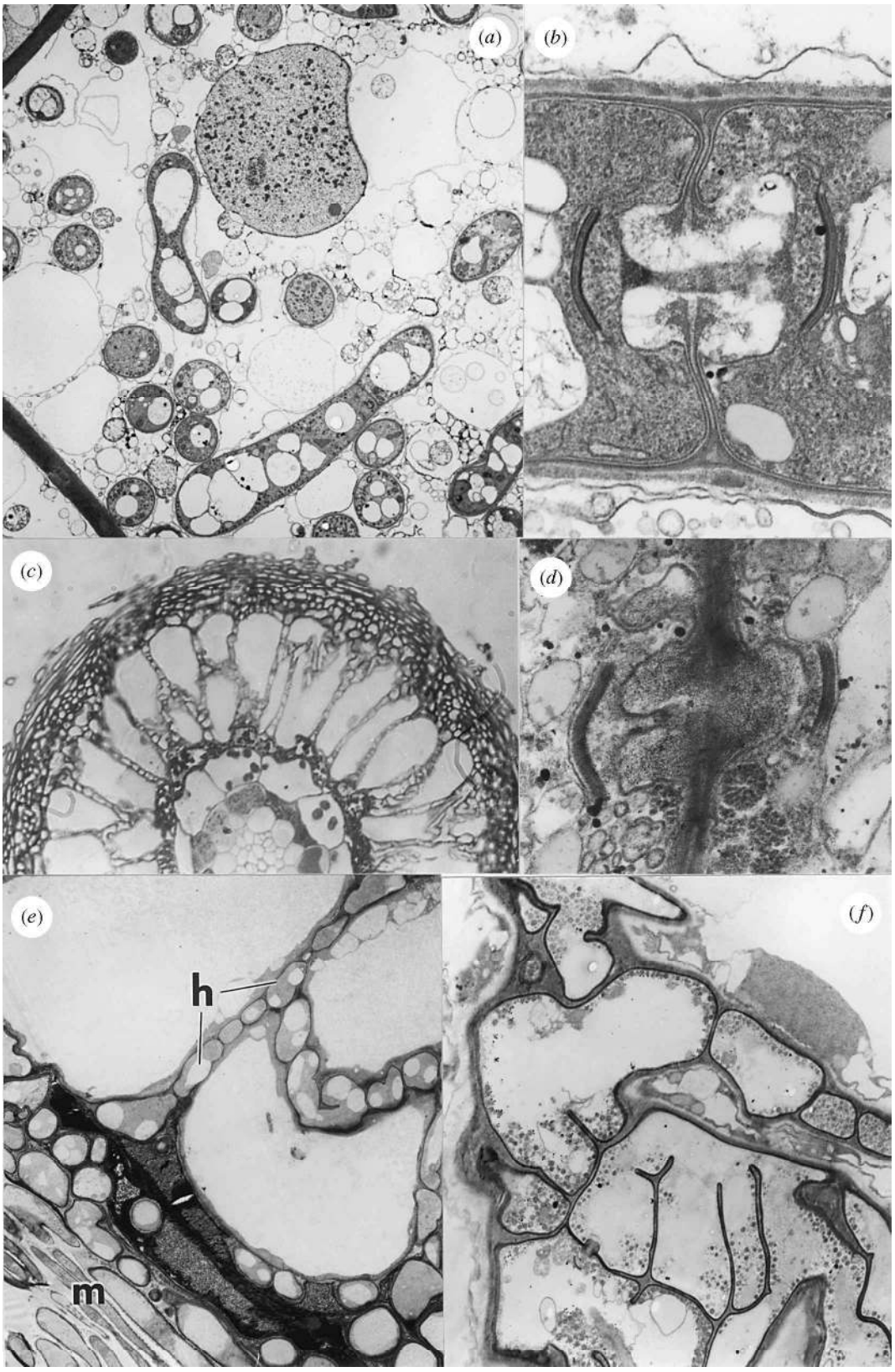

Figure 6. *Cryptothallus mirabilis*. (*a*) Inner thallus cell showing numerous hyphal profiles; × 3200. (*b*) Dolipore with imperforate parenthosomes; × 44 000. (*c–f*) Ectomycorrhiza in *Betula* formed by the *Cryptothallus* endophyte. (*c*) 1 μm toluidine-blue-stained section showing a typical mantle and Hartig net; × 580. (*d*) Dolipore of the same with the same morphology as in *Cryptothallus*; × 46 000. (*e,f*). Details of the mantle (m) and Hartig net (h). In (*f*) note pseudoparenchymatous nature of the fungus, a typical feature of Hartig nets; (*e*) × 2800; (*f*) × 6400.

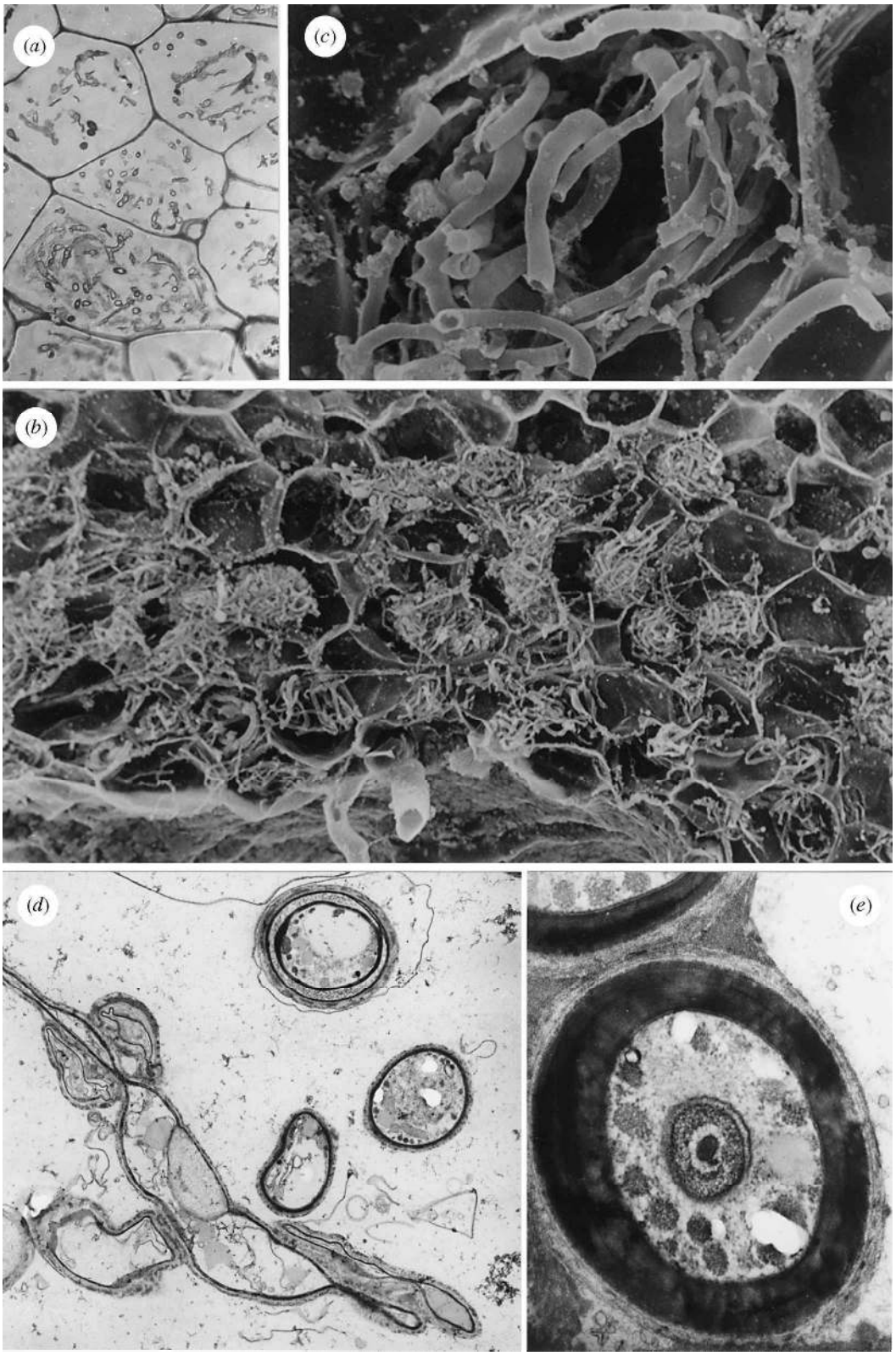

Figure 7. *Aneura pinguis*: resynthesized fungal association. (*a*) 1 μm toluidine-blue-stained section showing hyphal coils; × 470. (*b*,*c*) Scanning electron micrographs showing hyphal coils; (*b*) × 250, (*c*) × 1300. (*d*) Hyphae with multilamellate walls, a characteristic feature of the *Aneura* endophyte; × 8500. (*e*) Transverse section of a dolipore; × 40 000.

Read 1995). In cross inoculation experiments, the hepatic fungus isolated from *Cephalozia* and *Kurzia* produced typical ericoid mycorrhizas following the introduction of axenically cultured seedlings of *Calluna*, *Erica* and *Vaccinium*. While the identification of the ascomycete isolated from the liverworts is yet to be confirmed, several species of leafy hepatics were readily infected by the ascomycete *Hymenoscyphus ericae*, originally isolated from the roots of *Calluna* (figure 4). Not only do these experiments considerably extend the known host range of *Hymenoscyphus ericae*, but the sharing of a common endophyte may also have considerable physiological and ecological impacts for

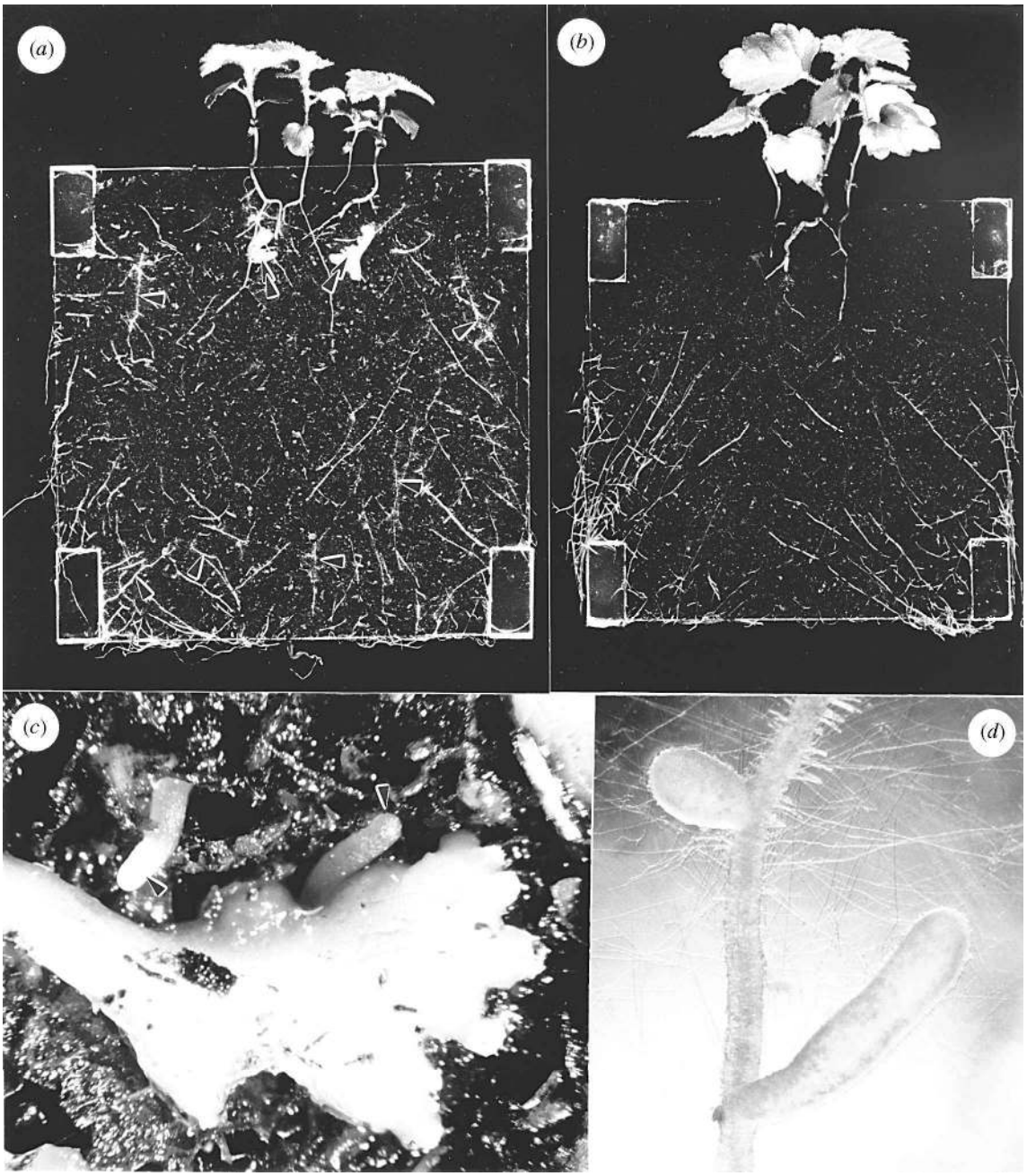

Figure 8. (*a*) Transparent Plexiglas microcosm supporting *Betula* seedlings growing on sterilized *Sphagnum* peat with plants of the achlorophyllous hepatic *Cryptothallus mirabilis* (double arrows). Mycorrhizal fungal hyphae (single arrows) grow from *C. mirabilis* to colonize the peat. (*b*) A parallel microcosm with *Betula* grown on the same medium as in (*a*) but without *Cryptothallus*. Note absence of fungal mycelia. (*c*) Close-up view of *C. mirabilis* thallus grown with *B. pendula* as in (*a*) showing the conversion of roots of *Betula* to ectomycorrhizas (single arrows) in the vicinity of the hepatic. (*d*) Ectomycorrhizal laterals formed on monoxenically grown *Betula* seedlings inoculated with a pure culture of the mycorrhizal fungus of *C. mirabilis*. Sections of such roots (see figure 6*c*) reveal the typical ectomycorrhizal structures, a Hartig net and mantle.

both the liverwort and ericaceous hosts. We are currently investigating carbon exchange and nutrient relationships using a similar approach to the studies with VA and basidiomycete associations.

(iii) *Basidiomycetous associations in hepatics*

In contrast to the Cephaloziaceae, other leafy hepatics are colonized by endophytes inhabiting a discrete region of the inner stem. This type of association encompasses members of the Lophoziaceae, Arnelliaceae and Scapaniaceae (figure 5). The presence of dolipores in EM studies demonstrates, we believe for the first time, that the fungi involved are basidiomycetes. The hyphal coils seen within healthy host cytoplasm are reminiscent of those seen in orchid mycorrhizas.

Light and ultrastructural analyses of the achlorophyllous subterranean gametophytes of *Cryptothallus* and its closely allied photosynthetic relative *Aneura* have revealed closely similar cytology of robust coiled intracellular hyphae with dolipore septa (figures 6 and 7). This confirms the basidiomycete affinities of the endophytes suggested by Ligrone *et al*. (1993).

Little is known about the functions of these basidiomycete associations, but our recent discovery that the fungi from both *Aneura* and *Cryptothallus* can be grown axenically obviously opens the door to experimentation. To this end we have successfully reintroduced an *Aneura* fungal isolate of *A. pingus* into thalli of this species grown axenically from spores, thus fulfilling Koch's postulates (figure 7).

In view of its achlorophyllous nature, it is logical to hypothesize that the carbon requirements of *Cryptothallus* are supplied by its fungal symbiont. Until recently we had no knowledge either of the source of any such carbon or its method of transfer. That the plant has exacting habitat requirements is, however, well documented. It most commonly grows under *Sphagnum* lawns where there is an overstory of *Betula* (Paton 1999). Under these circumstances the source of carbon could therefore be either from the autotrophic associates or from the peat in which they are growing. Field observation indicating that birch roots growing in the vicinity of *C. mirabilis* plants were heavily colonized by ectomycorrhizal fungi led us to hypothesize that the hepatic was part of a tripartite association in which the fungus provided links to the tree. This hypothesis is being tested in a number of experiments. Aseptically produced seedlings of *Betula pendula* have been grown on thin layers of sterile *Sphagnum* peat in a series of transparent observation chambers. After the *Betula* root system had begun to extend across the peat, freshly collected, surface-washed thalli of *C. mirabilis* were planted into half of the chambers (figure 8*a*). Chambers with and without thalli were incubated in a controlled environment growth cabinet with only the *Betula* shoots exposed to light. While the birch plants without *Cryptothallus* produced no ectomycorrhizas, those in chambers containing the liverwort became heavily colonized by an ectomycorrhizal fungus (figure 8*b*). In a parallel experiment, the basidiomycetous fungus was isolated from *Cryptothallus* and grown in association with aseptically grown *Betula* seedlings using an agar-based Petri dish system developed by Brun *et al.* (1995). After four weeks of incubation, ectomycorrhizal roots were produced on the seedlings (figure 8*c*) confirming that the endophyte of *C. mirabilis* is an ectomycorrhizal fungus.

Sections of the birch roots from both experiments examined by LM and transmission electron microscopy (TEM) (figure 6) confirmed that the structures produced by the fungus, including a mantle and Hartig net, were those of a typical ectomycorrhiza. These observations clearly demonstrate that *Betula* and *Cryptothallus* can be interlinked structurally via a fungus but they do not, however, shed any light on the functional relationships between the partners.

To investigate the potential of *Betula* seedlings to supply *Cryptothallus* with carbon, a ^{14}C-based labelling study was carried out using tripartite systems set up in the transparent observation chamber. The results to date indicate that there is indeed a transfer of carbon from the autotrophic 'higher' plant to its heterotrophic 'lower' plant neighbour.

3. FUNGAL SYMBIOSES IN EXTANT LYCOPSIDA

The gametophytes of *Lycopodium* can be either subterranean and achlorophyllous, or surface-living with chlorophyll, depending on the species. It was established in classical early LM studies of their anatomy (Treub 1884, 1890*a*,*b*; Bruchmann 1885, 1910; Lang 1899, 1902; Burgeff 1938) that they were invariably invaded by fungi. According to the results of these studies, gametophytes of almost all species died at an early stage of development if they were not colonized by an appropriate fungus. More recently, there have been detailed ultrastructural analyses of a representative of the achlorophyllous types *L. clavatum* (Schmid & Oberwinkler 1993) and of a chlorophyll-bearing form *L. cernuum* (Duckett & Ligrone 1992).

The EM studies have confirmed the earlier descriptions of the fungus involved in the association as being aseptate, and this, together with the fact that the intracellular hyphae sometimes produce terminal vesicles, albeit of very small size, has led these symbioses to be placed in the VA category (Harley & Smith 1983; Harley & Harley 1987). It must be emphasized, however, that the taxonomic status of the fungi involved in lycopod gametophyte associations is not resolved. Schmid & Oberwinkler (1993) contrast the apparent lack of arbuscules, which is evident from the earlier LM as well as their own study, with the persistent presence of dense and strikingly regular coils of very fine hyphae having diameters in the range 0.8–1.8 μm. Such coils, again produced by extremely narrow hyphae, were also seen in *L. cernuum* gametophytes by Duckett & Ligrone (1992). The presence of coils rather than arbuscules is consistent with the notion of a '*Paris*-type' VA mycorrhiza as indicated above, and one fungus, *Glomus tenuis*, which produces associations generally accepted to be of the VA type, in higher plants, is characterized by having hyphae and vesicles of the very fine dimensions seen in *Lycopodium* prothalli (Hall 1977; Smith & Read 1997).

A further feature suggesting that the fungus may have glomalean affinities is that its intracellular hyphae, in both *L. clavatum* and *L. cernuum*, are occupied by 'bacterium-like' organelles (BLOs), which are indistinguishable from those described in hyphae of coarser VA endophytes (MacDonald & Chandler 1981; MacDonald *et al.* 1982). Apparently similar BLOs, now thought on the basis of molecular analysis to be of the *Burkholdera* type (Bianciotto *et al.* 1996) have been observed in putative VA endophytes of the hornwort *Phaeoceros laevis* (Ligrone 1988) and the thalloid hepatic *Conocephalum conicum* (Ligrone & Lopes 1989). They have also been reported in the *Glomus*-related zygomycete *Endogone flammicorona*, which forms ectomycorrhiza (Bonfante-Fasolo & Scannerini 1977) and in some free-living fungi (Wilson & Hanton 1979).

There are some differences between the infections described in *L. cernuum* and *L. clavatum*. In the former, vesicles were not seen and, in addition to the predominantly fine hyphae, there were some of wider diameter, which were construed as being the trunks of arbuscules. Their presence led Duckett & Ligrone (1992) to hypothesize that there may be two types of infection in the same host genus, though they acknowledged that if arbuscules were formed they would be an unusual feature in *Lycopodium*.

On the basis of the distinctive nature of the structures seen in gametophytes of Lycopodiaceae, Schmid & Oberwinkler (1993) suggested that the association be

described as a 'lycopodioid mycothallus interaction' rather than as a mycorrhiza, and this caution may well be appropriate.

From the functional standpoint, the tacit assumption can be made that *Lycopodium* gametophytes, of the achlorophyllous kind at least, must be dependent on their fungus for carbon. Their failure to develop in the absence of colonization provides circumstantial evidence for this view. In this case, they can be regarded as 'dual organs of absorption' and accurately described as being mycorrhizal.

It is clear from the analyses of these symbioses made to date, however, that a disproportionate emphasis on structural studies has left us without resolution of most of the key questions concerning the homologies, taxonomic and functional, of fungus–lycopod associations. A search of the literature suggests that in only one study has an attempt been made, after appropriate surface sterilization, to isolate the fungus or fungi involved in the association in gametophytes and to reinoculate it to satisfy Koch's postulates. In this (Freeberg 1962), a fungus remarkably reminiscent of a *Rhizoctonia* was isolated. Prothalli grown with this fungus on a starch medium were found to gain weight more rapidly than those grown axenically. More studies of this kind are urgently needed. If the most important fungus turns out to have glomalean affinities it is likely that it will not, in fact, be culturable, but even in this event, molecular methods enabling characterization of these fungal taxa are now available and should be applied.

Since lycopod gametophytes can be readily obtained from nature (Duckett & Ligrone 1992) and grown *in vitro* (Whittier 1973; Whittier & Webster 1986), there should be no impedance to progress in this important area of research.

Analyses of the mycorrhizal status of the sporophyte generation of *Lycopodium* species have normally reported the presence of colonization by VA fungi (Boullard 1979; Harley & Harley 1987). This raises fascinating questions concerning the relationships, taxonomic and functional, between the fungi colonizing the heterotrophic gametophyte and autotrophic sporophyte stages of the life cycle. We return to this issue later.

4. FUNGAL SYMBIOSES IN EQUISETOPSIDA

There appears to be no record of any fungal association in the autotrophic gametophyte generation of *Equisetum*.

The question of the mycorrhizal status of the sporophyte generation has been the subject of some debate. The first reported analyses of *Equisetum* roots (Höveler 1892; Stahl 1900) revealed no fungal colonization. Subsequently Berch & Kendrick (1982) examined the root systems of numerous *Equisetum* species and also reported them to be largely free of colonization. On the basis of these results and an analysis of the early literature, Berch & Kendrick concluded that the genus was non-mycorrhizal. They ventured also to suggest that the decline of the Equisetopsida from the position of prominence which it occupied in Carboniferous and Jurassic forests was attributable to a failure to compete with mycotrophic neighbours. Subsequently Koske *et al*. (1985) have described what they call 'typical' VA mycorrhizas in several species of *Equisetum* growing in sand-dune systems. Conscious of the possibility that the presence of VA fungi in their roots might reflect simply the penetration of a 'non-host', attempts were made to collect samples from plants that were growing in 'isolated' positions. In fact, a distance of only 0.5 m was achieved from a nearest non-*Equisetum* neighbour, which, in view of the extensive root systems typical of mycotrophic plants, particularly grasses, in dune systems is unlikely to have achieved the desired effect. Their analyses led Koske *et al*. (1985) to postulate that the demise of *Equisetales* was attributable to a worldwide change from hydric to mesic conditions which favoured other groups and was not related to a genetically determined inability to form VA mycorrhiza.

This debate highlights the problems arising from analyses based purely on the presence or absence of fungal structures. Clearly *Equisetum* grows perfectly well in the absence of mycorrhizal fungi under many circumstances. When associations between *Equisetum* roots and VA fungi do occur, the only way to establish the nature of the relationship is by experiment. In the absence to date of any evidence for an absorptive role or of a contribution by the fungus to plant fitness there is no justification for referring to these associations as being 'mycorrhizal'. It follows that the phylogenetic significance of the presence or absence of colonization in this group remains a matter of conjecture.

5. FUNGAL SYMBIOSES IN PSILOTALES AND OPHIOGLOSSALES

The gametophytes of all species in both of these orders are achlorophyllous subterranean structures with endophytic fungal associations.

In the Psilotales they have been most extensively studied in *Psilotum nudum* (Dangeard 1890; Darnell-Smith 1917; Lawson 1918; Holloway 1939; Bierhost 1953; Boullard 1957, 1979; Peterson *et al*. 1981; Whittier, 1973). One type of colonization, produced by an aseptate fungus which occasionally bears vesicles, is consistently present. The fungus involved was originally referred to the chytridiaceous genus *Cladochytrium* and was considered to be parasitic. These views of the taxonomic and functional status of the fungus are no longer accepted. Indeed, since colonization by the same fungus seems to be a prerequisite for healthy development of gametophytes there is a prima facie case for considering the association to be of the mycorrhizal kind. The only published ultrastructural account of *Psilotum* gametophytes describes cortical cells occupied by dense coils of aseptate hyphae, some of which bear terminal vesicles (Peterson *et al*. 1981). A sequence of hyphal development and degeneration was observed in these cells but no arbuscules were seen. Light and ultrastructural studies have revealed very similar endophytic fungal morphology and behaviour in the gametophytes of *Botrychium* (Schmid & Oberwinkler 1994; Nishida 1956). There is much to indicate, therefore, that these are zygomycetous fungi and it seems reasonable to hypothesize that, as in the case of lycopods, *Psilotum* and *Botrychium* gametophytes are supported by '*Paris*-type' AM associations. The need to test this hypothesis by experiment is again evident. The only major structural distinction between the *Psilotum* and *Botrychium* associations and those described in *Lycopodium* (Duckett & Ligrone 1992; Schmid & Oberwinkler 1994) is that the hyphae of the

Psilotum and *Botrychium* fungi are coarse. Since the gametophytes of *Psilotum*, *Lycopodium* and *Ophioglossales* can now all be grown axenically (Renzaglia *et al.*, this issue), it should be possible to examine responses of the gametophyte to challenge by a range of glomalean fungi, with a view to establishing the basis of any relationship which they may have with these plants.

Peterson *et al.* (1981) and Schmid & Oberwinkler (1994) observed that many of the fungal hyphae and vesicle-like structures in *Psilotum* and *Botrychium* gametophytes stored lipids, which appeared to be released into the cortical cell cytoplasm on hyphal degradation. These authors speculated that the fungus may be able to synthesize lipids from soil organic matter but there is no evidence for such a pathway or for metabolism of fungus-derived lipid by the plant.

A relatively small number of observations on prothalli of *Tmesipteris* (Holloway 1917; Lawson 1918) suggest a similar type of infection. Spores of *T. elongata* have also been germinated axenically (Whittier & Given 1987).

The sporophytes of Psilotales and Ophioglossales are normally relatively massive autotrophic structures. While it is recognized that rhizomes and roots, respectively, are normally though not invariably, colonized by mycorrhizal fungi, it is necessary to emphasize at the outset that there is no evidence that infection spreads from the gametophyte into the developing sporophyte. Boullard (1963) observed that the rhizome of ophioglossaceous ferns was free of colonization and concluded that roots emerging from it were infected 'de novo' from soil. Likewise Mesler (1976) saw no fungal colonization of the embryo. The importance of these observations, which seem to parallel those in lycopods, lies in the fact that the two stages of the life cycle may be colonized by quite different fungi. This must be taken into account when considering carbon transfer into and out of the respective generations (see below).

Janse (1897) pointed out that rhizomes of *Psilotum* possessed hairs through which endophytic fungi passed to infect cortical cells, where they produced coils and vesicles. The fungi are aseptate and, in addition to vesicles and coils, arbuscules have also been seen (Burgeff 1938). It would thus seem appropriate to regard this association as being of the AM kind.

A broadly similar picture emerges from studies of *Ophioglossum* sporophytes though here arbuscules of a distinctive structure appear to be the norm. In *O. pendulum* Burgeff (1938) described single hyphae penetrating the host cells and branching profusely to produce many intracellular vesicles, which he called 'sternarbuskeln'. In the ultrastructural analysis of Schmid & Oberwinkler (1996), these swellings were shown to arise at the tips of what otherwise look like typical arbuscular branches. Some of these arbuscules arise directly from hyphal coils in the manner described in '*Paris*' mycorrhiza by Gallaud (1905). A further feature suggestive of AM affinities is the presence of bacteria-like organisms in the intracellular hyphae of *O. reticulatum* (Schmid & Oberwinkler 1996).

Ascending finally to 'higher' ferns, here the green photosynthetic gametophytes are generally considered to be fungus-free. Light microscope studies do, however, describe the sporadic occurrence of endophytic fungi and suggest they may be constantly present in basal families like the Marattiaceae, Osmundaceae, Gleicheniaceae and Schizaeaceae (Boullard 1979; Campbell 1908; Bower 1923). A recent LM and EM study (Schmid & Oberwinkler 1995) describing arbuscules and lipid-packed vesicles in healthy gametophyte cells of Gleicheniaceae concludes that the association is closely similar to the infection in *Conocephalum* and *Phaeoceros*. As with every other pteridophyte association, investigation of function is now required.

6. CONCLUSION

From the analyses presented above it becomes obvious that a large discrepancy exists between knowledge of the occurrence of fungal symbioses in 'lower' plants and our understanding of their functions. Even in those cases such as the achlorophyllous gametophytes of lower tracheophytes, where it seems logical to hypothesize a function based on carbon supply by the fungus, fundamental questions remain. What is the source of this carbon? Are the same fungal taxa colonizing the heterotrophic gametophyte and the autotrophic sporophyte? If so, how is the switch in polarity of carbon movement achieved?

Similar problems confront us when attempting to hypothesize functions for fungal symbioses in poikilohydric leafy hepatics. Should we envisage that nutritional advantages of the kind known to accrue to homoiohydric 'higher' plants from mycorrhizal colonization will necessarily be observed in slow-growing poikilohydric liverworts? It is reasonable to hypothesize that the development of such plants is rarely, if ever, limited by nutrient availability. This symbiosis could arise simply because selection has favoured loss of specificity in these fungal biotrophs as it maximizes their access to carbon. A 'mycocentric' view of this kind would place many of the associations described above at the level of commensal rather than mutualistic symbiosis, thus weakening any argument in favour of them being similar to mycorrhizas. Again, experiment alone will resolve these issues. In view of the consistency with which each of the described types of colonization occurs in its respective group of 'lower' plants, it seems reasonable to hypothesize that physiological interactions of importance to both partners must occur. There is an urgent need to test such hypotheses.

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Discussion

A. E. Newton (*Department of Botany, Natural History Museum, London, UK*). Following up on the statement that mosses lack fungal associations, while many other lower plants (hepatics, anthocerotes, early fossils, etc.) have been found to have several different types of symbiotic association: (a) Which taxa of mosses have been studied? and (b) Why do you think mosses lack these associations?

J. G. Duckett. I have examined over 200 taxa throughout the mosses.

D. J. Read. Response to (a) above. Mosses may not have been systematically searched for the presence of fungal symbionts. However, the view that they lack fungal associations is based on extensive observations carried out over many years by bryologists on the one hand and those interested in mycorrhizas on the other.

D. J. Read. Response to (b) above. In the absence of experimental analysis of the responses of mosses to challenge by fungal symbionts, it is possible to speculate that some feature of the wall structure of moss cells renders them impenetrable. Mosses are hosts to remarkably few fungal pathogens and are also generally unpalatable to herbivores, both features being indications of effective 'defence' against intrusion.

J. G. Duckett. Response to (b) above. Mosses possess extensive protonemal/rhizoidal systems that may mimic the fungal systems, making the fungal association unnecessary. This is turn raised the question 'What is the situation in *Andreaea* and *Andraeaobryum*, taxa that lack rhizoidal systems?'

D. J. Read. We have looked at both *Andreaea* and *Andraeaobryum*—they are never infected. However, I would not use the 'rhizoid'-based response of Jeff. Both liverworts and mosses have rhizoids. Indeed, in liverworts it is the rhizoids that provide the channels for penetration and in many cases they harbour the fungal colonies.

P. Kenrick (*Department of Palaeontology, Natural History Museum, London, UK*). You have discussed fungus associations of terrestrial plants rooted in soil and shown us some striking examples of how the absence of a fungus under experimental conditions affects the vigour of the plant. Do epiphytic and epilithic plants also have fungal associations?

D. J. Read. The patterns of fungal symbiont colonization seen in hepatics of normal terrestrial habitats are repeated in epiphytic and epilithic situations.